Title : Cod uracil-DNA glycosylase, gene coding therefore, recombinant DNA containing said gene or operative parts thereof, a method for preparing said protein and the use of said protein or said operative parts thereof in monitoring
5   or controlling PCR.

Field of Invention

The present invention relates to a novel enzyme present in cod liver, a DNA sequence encoding the enzyme or operative - or biologically active parts
10   thereof, a novel recombinant DNA comprising the gene or the operative parts thereof, a method of preparing the enzyme from cod liver and from bacteria carrying the gene, the bacteria carrying the gene *per se*, and the use of the protein in monitoring and/or controlling PCR or related reaction systems.

15   **Description of prior art**

Uracil in DNA may result from the incorporation of dUTP instead of dTTP during replication or from deamination of cytosine in DNA. The latter results in a mutation at the next round of replication. The enzyme Uracil-DNA glycosylase (UDG, EC 3.2.2.3) functions as a repair enzyme for such damage to DNA in
20   that the uracil base is excised from the DNA backbone (Lindahl 1994), creating an apyrimidinic site which is recognised by other DNA repair enzymes. This enzyme is crucial to maintain DNA integrity, and has therefore been found in a variety of organisms; virus, prokaryotes and eukaryotes.

25   Uracil-DNA glycosylase (UNG or UDG) catalyses the hydrolysis of the N-glycosylic bond between the deoxyribose sugar and the base in uracil-containing DNA, and was first isolated and characterized from *Escherichia coli* (1). This is the first step in the base excision repair (BER) pathway of removing uracil from DNA (2), and the aprymidinic site generated is thereafter repaired by
30   an AP endonuclease, phosphodiesterase, DNA polymerase and DNA ligase (other enzymes) in the BER pathway (3-5).

Several classes of uracil-DNA glycosylases (UDG) have been described. The major cellular form of UNG is UNG encoded by the UNG-gene (6). Other classes comprises the cyclin-like human UDG2 (7,8), single-strand selective monofunctional UDG SMUG1 from human and *Xenopus* (9), G/T:U specific .

5   mismatch DNA glycosylase (MUG) isolated from *E. coli* (10,11) and *Thermotoga maritima* UDG (TMUDG) (12).

UNG is a monomeric protein, about 25-35 kD in size. It is not dependent of any cofactors or divalent cations and is highly conserved among different species

10   (13). UNG is, however, affected by ionic strength. The UNG enzyme has been shown to act in a processive "sliding mechanism", where it locates sequential uracil-residues prior to dissociation from DNA (14,15), and a distributive "random hit" mechanism (16). UNG has previously been isolated and characterised from rat liver (17-19), human cells and tissues (20-28), calf

15   thymus (29,30), slime mold (31), yeast (32), plants (33,34), brine shrimp (35), procaryotes (1,36-45) and viruses (46,47).

In human and rat both a mitochondria and nuclear UNG have been isolated (18,25). In human (and mouse) cells these two are encoded by the same gene

20   (UNG) (48,49). By two different transcription start sites and alternative splicing, two forms are generated which differ only in the N-terminal signal sequence, which targets the enzyme to the nucleus (UNG2) and mitochondria (UNG1), respectively (49). Recently, several studies have been done to further study the N-terminal signal sequence and targeting of UNG to the nucleus and

25   mitochondria (50-52), revealing the nuclear UNG2 to be phosphorylated (26). The crystal structures of UNG from human, herpes simplex virus and *E. coli* have been solved (53-55). The active site residues are conserved and the mode of action in these enzymes seems to be the same with a nucleotide-flipping mechanism to remove uracil from DNA (56,57).

30

Enzymes from cold adapted organisms, such as the Atlantic cod (*Gadus morhua*), have to compensate for the reduction of chemical reaction rates at low temperatures in order to maintain sufficient metabolic activities. This can be

achieved by higher transcriptional/translational levels or improved catalytic efficiency (k_{cat}/K_M). Higher catalytic efficiencies can be reached by a more flexible structure, compared to their mesophile counterparts, which provides enhanced ability to undergo conformational changes during catalysis. The

5 reduced stability to pH, temperature and denaturing agents is regarded as a consequence of the conformational flexibility (58).

The present invention concerns the purification and characterization of a heat-labile uracil-DNA glycosylase from a cold-adapted organism, and which has

10 utility as an enzyme efficient in carry-over prevention in DNA-copying reactions (PCR, LCR etc.). The enzyme isolated according to the present invention has similar characteristics as previously described UNGs with respect to molecular weight, isoelectric point, pH and NaCl-optimum. However, the enzyme according to the present invention has been shown to be more pH-labile and

15 heat-labile and has a higher relative activity at low temperatures, compared to a recombinant mesophilic human UNG, making it a better candidate in carry-over prevention tests as indicated *supra*.

UNG (or UDG) from *Escherichia coli* has been commercially available for use in

20 carry-over prevention when amplifying DNA material.

Various techniques may be employed to amplify specific DNA sequences on basis of a DNA template. Common techniques are the polymerase chain reaction (PCR) system (US patent Nos. 4.683.195; 4.683.202; and 4.965.188),

25 the ligase amplification system (PCT patent publication No. 89/09835), the self-sustained sequence replication system (EP No. 329.822 and PCT patent publication No. 90/06995), the transcription-based amplification system (PCT patent publication No. 89/01050 and EP No. 310.229) and the Qβ RNA replicase system (US patent No. 4.957.858). These techniques are very

30 sensitive in that they may produce detectable DNA amounts from very few copies of a target DNA sequence. Accordingly the techniques are very sensitive to contamination by DNA from the environment. The major source of

contamination is products from previously performed up-scaling reactions, e.g. PCR r actions (59).

To overcome this problem a method to discriminate between target DNA and
5 contaminating DNA from prior reactions, e.g. PCR product DNA, has been
developed (60). In essence, all amplification reactions are carried out using
dUTP to replace dTTP thus incorporating uracil into DNA in place of thymidine.
All subsequent reaction mixtures are then treated with UDG (or UNG). A
following heat treatment degrades the contaminating DNA by hydrolysis of the
10 phospho diester bond at abasic sites. Also, the heat treatment is supposed to
inactivate the UDG enzyme.

However, the UDG (or UNG) enzyme from *E. coli* is not completely inactivated
by heat treatment, and the inactivation is not completely irreversible (60). This
15 affects the upscaling reaction, e.g. PCR reaction, product integrity, since the
products are degraded by residual UDG enzyme activity. In order to avoid this,
the subsequent addition of enzyme inhibitor to UDG has been used (US Patent
No. 5.536.649).

20 It would however, be preferable to avoid using the subsequent inhibitor because
this represents an extra step in the reaction procedure, residual contamination
by the inhibitor may be present in subsequent reactions, and the purchase of
inhibitor represents an extra cost when performing a PCR reaction as disclosed
supra.
25

Thus it would be preferable to use an UNG (or UDG) enzyme which is certain to
be destroyed/inactivated by the heat treatment following the PCR reaction, thus
avoiding the addition of specific chemical inhibitors for the UDG enzyme.

30 **Closer description of the figures**
Figure 1 shows pI determination of Atlantic cod UNG (cUNG). Following the
isoelectric focusing using Phast Gel IEF 3-9, the gel was cut in pieces of 2 mm.

Each piece was transferred to an eppendorf tube and incubated overnight at 4 °C. Activity was then measured as described in material and methods

Figure 2 shows product inhibition of cUNG with free uracil. Different
5   concentrations of uracil was added to the assay mixture and the assay performed as described in material and methods.

Figure 3 shows inhibition of cUNG with the *Bacillus subtilis* bacteriophage uracil-DNA glycosylase inhibitor (Ugi). 6.65×10^{-4} U of cUNG was incubated
10   with 1.25×10^3 U to 2.00×10^2 U of Ugi using standard assay conditions, as described in material and methods. One Unit of Ugi inhibit one Unit of UNG-activity, where the UNG-activity is defined as releasing 60 pmol of uracil per min at 37°C.

15   Figure 4 shows pH and NaCl optimum of cUNG. Activity of cUNG was measured with variable sodium chloride concentratios in different pH-series, as described in material and methods. The percent UNG activity is set relative to the highest value measured, pH 7.5 with 50 mM NaCl.

20   Figure 5 shows temperature optimum of cUNG. Due to prolonged incubation of the enzyme sample on ice during the experiment, activity is corrected with respect to the stability of the enzyme, as described in material and methods.

Figure 6 shows Temperature profile of cUNG (Λ) and rhUNG (◻). Enzyme
25   activity was measured as described in material and methods. The percent UNG activity is set relative to the highest value for cUNG (45°C) and rhUNG (50°C) respectively. Due to prolonged incubation of the enzyme samples on ice during the experiment, activity is corrected with respect to the stability of the enzymes, as described in material and methods.
30

Figure 7 shows pH stability of Atlantic cod UNG (cUNG) and recombinant human UNG (rhUNG). In the different pH-buffers, 1 U of cUNG (Δ) or rhUNG (◻) were incubated for 10 minutes at 37°C. Then 5 µl aliquotes were transferred to

the assay mixture, and residual activity was measured using standard assay conditions as described in materials and methods. One hundred percent activity was measured directly from a sample diluted at pH 8.0 without any incubation step.

5

Figure 8 shows temperature stability of Atlantic cod UNG (cUNG) (Δ) and recombinant human UNG (rhUNG) ($\square$).

Enzyme (1 U) were incubated at 50°C, 37°C, 25°C and 4°C, and 5 μl aliquotes

10 were transferred to the assay mixture after different time intervals, and standard assays were performed as described in material and methods. Half-lifes were determined to 0.5 min (50°C), 20 min (37°C), 60 min (25°C) and 2 h (4°C) for cUNG and 8 min (50°C) , 30 min (37°C), 150 min (25°C) and 2.6 h (4°C) for rhUNG.

15

Figure 9 shows agarose gel showing PCR products from carry over prevention test using recombinant Cod UNG (rcUNG). Lane descriptions: lanes 1 and 8: DNA ladder; lane 2: T-containing template, no UNG; lane 3: T-containing template, 1.7×10^{-3} U rcUNG; lane 4: U-containing template, 4×10^{-4} U rcUNG;

20 lane 5: U-containing template, 1.7×10^{-3} U rcUNG; lane 6: U-containing template, 4×10^{-4} U E. coli UNG; lane 7: U-containing template, no UNG.

Objects of the invention

It is an object of the present invention to provide a novel UNG (or UDG) enzyme

25 which is functional in carry-over prevention techniques for DNA amplification reactions, e.g. PCR, indicated supra, and which is completely and irreversibly inactivated by the heat treatment normally performed in PCR reaction cycles.

Furthermore it is an object of the present invention to provide a DNA sequence

30 coding for such an enzyme or an active part thereof, a vector or vector system (e.g. a virus, a plasmid, a cosmid, etc.) carrying such a DNA sequence and a micro organism including such a vector.

It is also an object of the present invention to provide a method for efficient production of the enzyme or an active part thereof by using genetic engineering techniques.

5   **Detailed description of the invention**

Enzymes from cold adapted organisms living in cold habitats, such as the Atlantic cod (*Gadus morhua*), have to compensate the reduction of chemical reaction rates at low temperatures to maintain sufficient metabolic activities. This can be done by a higher transcription/translational level, or improved
10   catalytic efficiency (k_{cat}/K_M). This higher efficiency is reached with a more flexible structure, compared to their mesophile counterparts, which provides enhanced ability to undergo conformational changes during catalysis. The reduced stability to pH, temperature and denaturing agents is regarded as a consequence of the conformational flexibility (58).
15

In view of the disadvantages with contaminations of UDG according to prior art in carry-over prevention procedures when amplifying DNA sequences (PCR, LCR) it would be very convenient to provide a UDG (or UNG) enzyme which was 100% degraded by simple heat treatment. It has now been found that a
20   UNG (or UDG) enzyme isolated from cod has this valuable property. The cod-UNG enzyme isolated according to the present invention has a similar molecular weight, isoelectric point, pH- and NaCl-optimum as previously described UNGs, but the present cod UNG is more pH- and heat-labile and has a higher relative activity at low temperatures as compared to a recombinant
25   mesophilic human UNG.

The enzyme according to the present invention has uracil-DNA glycosylase acitivity and is completely inactivated when heated above about 60 °C. It must be understood that this temperature treshold may vary within a range of a few
30   degrees.

Preferably the enzyme according to the invention has an amino acid sequence as shown in SEQ.ID.NOS:1 or 2, or a biologically functional part thereof.

The enzyme according to the pres nt invention is preferably derived from an organism adapted to a cold environment, more preferably the organism is eukaryotic, and most preferably the organism is Atlantic cod.

5   A DNA-sequence encoding for the novel enzyme as defined in the patent claims is another aspect of the present invention. Preferably the DNA-sequence has a nucleotide sequence as given in the SEQ.ID.NOS. 1 or 2.
The DNA sequence according to the present invention is preferably including a promoter and contained in an expression vector such as a plasmid, a cosmid or
10   a virus.

Further aspects of the invention is a micro organism comprising the said DNA-sequence of the invention as set out in the claims and use of the enzyme in monitoring and/or controlling a reaction system multiplying DNA-sequences
15   such as PCR or LCR. In particular the enzyme of the invention is used in a carry-over prevention procedure.

Infra is disclosed an isolation procedure tor the relevant cod UNG protein according to the invention, as well as isolation of DNA coding tor this protein
20   also its use in micro organisms for producing recombinantly the relevant UNG enzyme according to the invention, is disclosed.

Example 1

Extraction, purification and characterization of codUNG (cUNG)
25
Purification of cUNG

Preparation of crude extract and all purification steps were performed at 4°C. The following materials were used; Q-sepharose FF, S-sepharose FF, Heparin sepharose HP (Hi-trap 5 ml), Poly-U-sepharose 4B, Superdcx 75 HR10/30,
30   Phast system and Phast IEF gels (3-9) and LMW gel filtration calibration kit were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Deoxy[5-^{3}H]uridine 5'-triphosphate (19.3 Ci/mmol) was purchased from Amersham (U.K). Uracil-DNA glycosylase inhibitor (Ugi) was obtained from New England Biolabs (Bev rly, MA), enzymes were purchased from Promega
35   (Madison, WI). Proteas inhibitors, calf-thymus DNA (D-1501), uracil,

deoxyuridine and deoxyuridine-monophosphate were purchased from Sigma (St. Lois, MO). All other reag nts and buffers were purchased from Sigma and Merck (Darmstadt, Germany).

5 **Preparation of crude extract**

To 600 ml extract buffer (25 mM Tris/HCl, 100 mM NaCl, 1 mM FDTA, 1 mM DTT, 10% glycerol, pH 8.0 (25°C) 200 g of fresh cod liver was added and homogenized in an Atomix homogenizer (MSE, England). Before homogenization, the following protease inhibitor mix was added to the extract

10 buffer: 1 mM PMSF, 1µM pepstatine, 1 µM leupeptine, 10 µM TPCK and 10 µM TLCK (final concentrations). The homogenate was centrifuged at 28,000 g for 15 min and the supernatant was filtered through glasswool. Finally glycerol was added to 30 % (v/v), and the cod liver crude extract was frozen at -70°C.

Q-sepharose Fast Flow

15 1 liter crude extract was diluted with 1 liter buffer A (25 mM Tris/HCl, 10 mM NaCl, 1 mM EDTA, 1% glycerol, pH 8.0) (Fraction 1). The sample was applied in two portions (1 liter each) on a Q-sepharose FF column (5.0/15), equilibrated with buffer A, and then washed with 250 ml buffer A, using a flow-rate of 10 ml/min. Proteins bound to the column were eluted with 200 ml buffer A + 1.0 M

20 NaCl, and the column was re-equilibrated with buffer A before the next part of the sample was applied, as mentioned above. The UNG-containing flowthrough and wash fractions from both two runs were pooled (Fraction 2, 2340 ml).

S-sepharose Fast Flow

25 Fraction 2 was applied to a S-Sepharose FF column (1.6/10) equilibrated in buffer A, flow rate 10 ml/min. The column was washed with 300 ml buffer A + 60 mM NaCl, and eluted using a 200 ml linear gradient of 0.06-0.4 M NaCl in buffer A, flow-rate 5 ml/min. UNG-containing fractions were pooled (55 ml) and dialyzed overnight in buffer A (Fraction 3).

30

Heparine sepharos High Perf rmance

Fraction 3 was applied to a h parine sepharose HP Hi-Trap column (1.6/2.5) equilibrated in buffer A. Thc column was washed with 50 ml buffer A + 60 mM NaCl and was eluted in a 50 ml linear gradient of 0.06-0.4 M NaCl in buffer A,

5 flow-rate 1 ml/min. UNG-containing fractions were pooled (Fraction 4, 20 ml).

Poly-U sepharose (4B)

Fraction 4 was then diluted 5 times in buffer A, and applied to a poly-U sepharose column (1.6/10) equilibrated in buffer A. I he column was washed

10 with 60 ml buffer A + 60 mM NaCl and was eluted In a 200 ml linear gradient of 0.06-0.4 M NaCl in buffer A, flow-rate 1ml/min. UNG- containing fractions were pooled (fraction 5, 70 ml).

Superdex75

15 Fraction 5 was concentrated to 200 μl using Ultrafree15 and Ultrafree-MC ultracentrifugation filters (Millipore), cutoff 5K, and applied on the gel filtration column (HR 1.0/30) equilibrated in buffer A, with a flow-rate of 0.5 ml/min: Fractions (350 μl) were collected, and those containing UNG-activity were pooled (fraction 6, 3 ml).

20

Preparation of substrate by nick-translation

[3]H-dUMP DNA was prepared by nick-translation and polymerase chain reaction (PCR). The nick-translated substrate was made in a total volume of 1 ml and contained 50 mM Tris/HCl, pH 7.2, 10 mM $MgSO_4$, 1 mM DTT, 250 μg calf

25 thymus DNA (purified by phenol/cloroform extraction and ethanol precipitated prior to use), 0.1 mM dATP, dCTP, dGTP and dUTP, where 3 μM of the dUTP was [3H]-dUTP (19.3 Ci/mmol). Then 0.1ng (5.35×10^{-4} U) DNase I (bovine pancreas, Promega) was added and 30 seconds later 25 U of *E. coli* DNA polymerase, and the nick-translation mix was incubated at 21°C for 24 hours.

30 The nick-translated DNA was purified by phenol/chloroform extraction, and ethanol precipitation. DNA was resuspended in 50 μl TE-buffer and purified with a NAP-5 column (AP Biotech) equilibrated in TE-buffer (10 mM Tris/HCl, 1 mM

EDTA, pH 8,0) to remove unincorporated nucleotides. Specific activity of the nick-substrate was 1.8×10^5 dpm/µg (425 cpm/pmol).

Preparation of substrate by PCR

5   The PCR-produced substrate was used for all characterization experiments and consisted of a 761 bp fragment generated from cationic trypsinogen (sstrpIV) from Atlantic salmon (*Salmo salar*) (61). The PCR was carried out in a volume of 50 µl in a Perkin Elmer Cetus thermocycler. The PCR-mix contained 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 6 mM $MgCl_2$, 0.37 mM dATP, dCTP, dGTP and

10   dUTP, where 10.4 µM of dUTP was [³H]-dUTP (17.0 Ci/mmol, Amersham), 700 pg template DNA (sstrpIV in a pgem7zt-vector), 2.5 µM of upstream and downstream primers and 2 U Taq polymerase (Roche, Switzerland). The PCR-reaction was done by 30 cycles of 94°C for 1 min, 45°C for 1 min and 72°C for 1 min. Then an additional 2 U of Taq-polymerase was added and the PCR-

15   reaction continued with 30 new cycles as described. The PCR-substrate was purified with QIAquick PCR-purification kit (Qiagen) as described by manufacturer, and eluted in 50X diluted TE-buffer, pH 8.0. Specific activity of the PCR-substrate was 5.9×10^5 dpm/µg (451 cpm/pmol). All characterization experiments were done using the PCR-substrate.

20

Detection of uracil-DNA glycosylase activity (Standard assay)

Uracil-DNA glycosylase activity was measured in a final volume of 20 µl, containing 70 mM Tris/HCl, 10 mM NaCl, 1 mM EDTA, 100 µg/ml BSA and 230 ng nick-substrate or 71 ng PCR-substrate. The reaction mixture was incubated

25   10 min at 37°C, and terminated with the addition of 20 µl of ice cold single stranded calf thymus DNA (1 mg/ml) and 500 µl 10% (w/v) TCA. Samples were incubated on ice for 15 min, and centrifuged at 16,000 g for 10 min. Supernatants with acid soluble ³H-uracil were analyzed using a liquid scintillation counter.

30

On unit of activity is defined as the amount of nzyme required to release 1 nmol of acid soluble uracil per minute at 37°C.

Analysis of assay products with thin layer chromatography

Reaction products after assays were mixed with 20 nmol uracil, deoxyuridine and deoxyuridine- monophosphate. Thin layer chromatography was performed
5 according to the method by Wang and Wang, using polyamide layer plates (BDH), and tetrachloromethane, acetic acid and acetone (4:1:4, by volume) as solvent (62). Spots detected under UV-light were cut out of the plate and radioactivity measured in a liquid scintillation counter.

10 ## Molecular-weight determination

The molecular weight was determined by gel-filtration, and was performed with a Superdex 75 column (1.0/30) equilibrated in a buffer containing 25 mM Tris/HCl, 1.0 M NaCl, 1 mM EDTA, 1 % glycerol, pH 8.0. The flow-rate was 0.5 ml/min, and activity was measured in the fractions collected (250 µl). Bovine
15 serum albumin (BSA, 67 kD), ovalbumin (43 kD), chymotrypsinogenA (25 kD) and ribonucleaseA (13.7 kD) were used as standards. Blue dextran and sodium chloride were used to determine void- (V_0) and intrinsic volume (V_i), respectively.

20 ## Protein determination

Protein concentrations were determined with Coomassie® Protein Assay Reagent G-250 (Pierce, New York, NY) by the method of Bradford (63), with the microtiter plate protocol as described by manufacturer, using bovine serum albumin (BSA) as a standard.
25

Isoelectric point determination

Isoelectric point determination was done with the Phast-system, isoelectric focusing gel 3-9 and silver stained according to methods described by manufacturer. Standards used were phycocyanin (4.45, 4.65, 4.75), β-
30 lactoglobulin B (5.10), bovine carbonic anhydrase (6.00), human carbonic anhydrase (6.50), equine myoglobin (7.00), human hemoglobin A (7.10), human hemoglobin C (7.50), lentil lectin (7.8, 8.0, 8.2), cytochrome c (9.6), (IEF Standards pI range 4.45-9.6, BIO-RAD). After focusing, the gel was cut into 2

mm pieces and each incubated in 250 μl extraction buffer (50 mM Tris/HCl, 0.2 M NaCl, 1 mM DTT, 1 mM EDTA, 1 % (v/v) glycerol, pH 8.0 overnight. Aliquotes of 5 μl were transferred to the assay mixtures, and activity measured using standard assay conditions.

5

Determination of pH/NaCl-optimum

Assays were done in a volume of 20 μl as described in standard assay using the PCR generated substrate and NaCl concentration from 0-200 mM with 25 mM intervals, and pH ranging from 9.5-6.5 with 0.5 pH unit intervals. All buffers
10 were supplemented with 100 μg/ml BSA and 1 mM EDTA. The buffers used were diethanolamine/HCl (9.5-8.5), Tris/HCl (8.5-7.5) and MOPS/NaOH (7.5-6.5). All buffers were pH adjusted at 37°C and used in 25mM concentration in the assay.

15 ## Determination of temperature optimum

Assays were done in a volume of 20 μl as described in standard assay using the PCR generated substrate. The assay mixtures were as described in standard assay conditions and were adjusted to pH 8.0 for all temperatures. The temperature range used was 5°C to 60°C. The activity was measured in a
20 sequential manner with 15 min intervals between each temperature. The enzymes used were diluted in standard dilution buffer (5 mM Tris/HCl, 10 mM NaCl, 1 % (v/v) glycerol, pH 8.0) and placed on ice. Due to the instability of the enzyme sample on ice over a prolonged period, results were corrected with respect to the stability of the enzymes incubated in dilution buffer on ice, with
25 the formula $N_{(t)} = cpm / c^{-0.693(t/\lambda)}$, where half-life ($\lambda$) of cUNG and rhUNG are 2.0 hours and 2.6 hours respectively.

Effect of pH and temperature on stability

pH: UNG (0.01 U) was preincubated (in a total volume of 75 μl) for 10 min at
30 37° in buffers containing 10 mM buffer, 10 mM NaCl, 1 mM EDTA, 1% glycerol, with pH ranging from 9.5-6.5 with 0.5 pH unit intervals using diethanolamine/HCl (9.5-8.5), Tris/HCl (8.5-7.5), MOPS/NaOH (7.5-6.5) and

MES/NaOH (6.5-5.5) as buffer components. Aliquots of 5 µl were transferred to the assay mixtures and residual activity was measured using standard assay conditions.

Temperature : UNG (0.01 U) was preincubated (In a total volume of 75 µl) in 10
5 mM Tris/HCl, 50 mM NaCl, 1mM EDTA, 1% glycerol, pH 8,0 (adjusted at each temperature). After different time intervals, as Indicated in figure legends, 5 µl aliquots were transferred to the assay mixtures and residual activity was measured using standard assay conditions.

10 **Substrate specificity against ss/ds DNA**

PCR and nick-translated substrate was incubated 3 min at 100°C and thereafter rapidly cooled on ice to generate ssDNA. Following denaturation, the ssDNA-substrates were used in standard assay condition with 6.65×10^{-4} U purified cUNG. Enzyme activity was also measured using dsDNA substrates for both
15 nick- and PCR-substrate, and used as references.

Ugi inhibitor- and uracil product Inhibition

Activity measurements using PCR-substrate were performed with 6.65×10^{-4} U of purified cUNG. Various concentrations of uracil (0, 1, 2 and 5 mM) or Ugi-
20 inhibitor ($1,25 \times 10^3$ U to $2,00 \times 10^2$ U) were added to the assay mixtures (on ice). Activity was then measured as described under standard assay conditions.

RESULTS

Purification of cUNG
25 Atlantic cod UNG was purified 17,000 fold with a recovery of 2%, as shown in table 1. Despite the high purification factor the enzyme was only partly purified, as determined by SDS-PAGE. Also the yield was low, due to many purification steps, and the concentration of the dilute protein sample before the gel-filtration step.

30

Molecular weight and pI-determination

The molecular weight was determined by g l filtration to be 25 kD ± 2, from three separate experiments. The isoelectric point determination was done with

an IEF Phast Gel with IEF standards ranging from 4.42 - 9.6. Following IEF, cUNG activity was eluted from the gel fragments and activity measured as described in material and methods. cUNG activity co-eluted with the cytochrome c, standard with an isoelectric point of 9.6, as shown in figure 1.

5   The cytochrome c and cUNG activity were found where the electrode contacted the gel, therefore we can only conclude that the pI is larger than 9.0, which is the highest measurable value using this system.

Substrate specificity

10   cUNG activity was measured using both ssDNA and dsDNA. A 1.8 and 1.9 fold higher activity for ssDNA than dsDNA was found using nick- and PCR-substrates, respectively, as shown in table 2. Assay products were analyzed by thin layer chromatography, and the major part of the radioactivity was identified as uracil. However, some radioactivity was also co-localized with the

15   deoxyuridine marker, but this could be due to the partial overlap of the two markers. In addition the purified cUNG did not exhibit any significant hydrolysis of ^{3}H-adenine-labelled DNA, therefore excluding nucleases as responsible for hydrolyzing the DNA.

Inhibition studies

20   Product inhibiton by free uracil was examined, and gave more than 50 % inhibition with 1 mM uracil in the assay mixture, as shown in figure 2. Adding 5 mM free uracil to the assay mixture, a 78 % inhibition of the activity was observed. The effect of Ugi on cUNG was measured by adding Ugi to the assay

25   mixture. cUNG was clearly inhibited by Ugi, as shown in figure 3.

pH and NaCl optimum

The pH- and sodium chloride optimum was examined by measuring the enzyme-activity at different pHs using NaCl-concentration from 0-200 mM, as

30   shown in figure 4. The enzyme exhibited a broad pH-optimum, with maximal activity between pH 7.0-9.0, and 25-50 mM NaCl. A shift in NaCl optimum was observed, where the optimum NaCl concentration changed from low

concentrations at high pH to higher concentrations at low pH. At pH 9.5 cUNG was inhibited by NaCl.

Temperature optimum

5   The temperature optimum of cUNG was determined to 41°C (figure 5). To compare the activity of cUNG with the mesophilic rhUNG at low temperatures, enzyme activity was measured from 5-60 °C, and the activity at low temperatures compared to their respective optimum temperature (figure 6). The activity profile of these two enzymes showed little difference at 5-15°C.

10   However at temperatures from 20-40°C, a higher relative activity was observed with cUNG than rhUNG, whereas at high temperatures (50-60°C) the opposite was observed.

Stability

15   The stability of the two UNG enzymes were compared by preincubating the enzymes at different pHs. Atlantic cod UNG was shown to be most stable . between pH 7.0-8.5. At pH 5.5 and pH 10.0 it had less than 1 % residual activity. rhUNG was most stable between pH 7.0-9.5. At pH 5.5 3 % residual activity remained, but at pH 10.0, as much as 66% of the activity remained, as

20   shown in figure 7. The temperature stability of the two UNG-enzymes was compared at 4°C, 25°C, 37°C and 50°C. At 50°C, the half-life was determined to be 0.5 min and 8 min for cUNG and rhUNG respectively. At all temperatures examined, rhUNG was more stable than cUNG. Half-lives determined were 20 min (37°C), 60 min (25°C) and 2 h (4°C) for cUNG and 30 min (37°C), 150 min

25   (25°C) and 2.6 h (4°C) for rhUNG, but the largest difference in half-life was found at the highest temperature, as shown in figure 8.

DISCUSSION

Purification and molecular weight

30   The uracil-DNA glycosylase from Atlantic cod liver (Gadus morhua) was purified 17,679 fold using several chromatographic techniques. Still the enzyme was only partly purified, as several other bands were seen on a SDS-PAGE gel.

Human nuclear and mithochondrial uracil-DNA glycosylases are shown to be generated by alternate splicing, and have an ORF of 313 and 304 amino acids respectively (49). The molecular weight of cUNG was determined to 25 kD. This is approximately the same molecular weight as determined for the UNG

5 from human placenta (29kD) and the rhUNG (UNGΔ84) (27kD), which lacked 77 and 84 of the first N-terminal amino acids respectively, as predicted from the mithochondrial ORF (21,64). This suggest that the N-terminal signal sequence in the purified cUNG is processed or artificially cleaved during purification or that Atlantic cod UNG does not have a N-terminal signal sequence. During

10 purification we did not see any sign of two different UNGs as previously described during purification of UNG from rat or human sources (18, 23). But as a vertebrate, one should expect that Atlantic cod possesses both a nuclear and a mitochondrial form of UNG, but so far no effort has been given to reconcile this matter.

15 The uracil-DNA glycosylase from Atlantic cod was similar to other uracil-DNA glycosylases previously purified and characterized with respect to the high pI (19) and the two-fold preference to ssDNA than dsDNA (19).

Inhibition by Ugi and uracil

20 The *Bacillus subtilis* bacteriophage PBS2 UDG-inhibitor (Ugi) inhibits UNG by forming a stable complex with UNG at physiological conditions). Ugi binds to human UNG by inserting a beta-strand into the conserved DNA binding groove (67), and acts by mimicking DNA (68). This indicates that the structure of the substrate binding site of cUNG is similar to other UNGs inhibited by the Ugi-

25 inhibitor. Inhibition with free uracil was in agreement with values previously reported (19).

Optimum conditions

cUNG was shown to have a broad pH optimum from 7.0-9.0, and the activity

30 was strongly affected by NaCl-concentration. The broad optimum activity is previously reported for several other UNGs characterized (23, 31, 40, 47). Interestingly the NaCl-optimum for cUNG increases as pH decreases. It has previously been demonstrated that UNG functions in a processive manner at

low ionic strength, which involves that when NaCl-concentration increases, the enzyme switches to a distributive mechanism (14, 15). However Purmal et al reported the opposite, that UNG acted in a distributive mechanism at low ionic strength. The processive mechanism is a common feature among several DNA

5 Interactive proteins (polymerases, repressors, restriction/ modification enzymes, DNA repair enzymes), and the interactions are generally electrostatic in nature (67-71)]. In a UV-endonuclease from *Micrococcus luteus* the processive mechanism has also been shown to be pH-dependent (72). Therefore we suggest that the shift in NaCl-optimum with increasing pH reflects the

10 processive/distributive nature of cUNG. And it could be that the controversy in the previous reports are also due to different buffer components and pH, in addition to the differences already discussed.

The temperature optimum (41°C) was found to be slightly lower than the

15 mesophilic rhUNG (45°C) (64). The relative activity at temperature from 5-45°C was higher for cUNG than rhUNG. At temperatures higher than 45°C, rhUNG shows a higher relative activity than cUNG, presumably as a consequence of the low temperature stability of cUNG.

20 **Temperature and pH stability**

Enzymes characterized from cold-adapted species have been found to be more temperature and pH-labile, proposed due to their flexible structure in order to maintain enzymatic activity at low temperatures (73). cUNG was found to be both more pH- and temperature labile than the rhUNG, which are known

25 features for other cold-adapted enzymes (73, 74).

A psychrophilic UNG from a marine bacterium has previously been isolated, and was compared to *E.coli* UNG with respect to temperature stability (45). This procaryotic UNG had a half-life of 2 min at 40°C and 0.5 min at 45°C, compared

30 to 27 and 8 min for the *E.coli* UNG. cUNG was compared to the rhUNG with respect to both temperature and pH stability. At 50°C rhUNG had a half-life of 8 min, compared to 0.5 min for cUNG. At lower temperature the difference in half-life was less, although rhUNG was more stable than cUNG at all temperatures

examined. Both enzymes were shown to be labile at low pH, whereas at high pH rhUNG was more stable than cUNG.

In conclusion, the uracil-DNA glycosylase from Atlantic cod was shown to be similar to other uracil-DNA glycosylases previously purified and characterized with respect to molecular weight, high pl, a broad pH-optimum and a two-fold preference to ssDNA than dsDNA. However the cUNG was shown to be more temperature and pH-labile and has a higher relative activity at low temperatures than the mesophilic rhUNG.

Example 2

Isolation of the uracil-DNA glycosylase gene from *Gadus morhua*

The following materials were used; SuperScript™ II Rnase H⁻ Reverse Transcriptase (Gibco BRL), Packagene® Lambda DNA packaging system was purchased from Promega (Madison,WI), Deoxy[5-^{3}H]uridine 5'-triphosphate (17.0 Ci/mmol) was purchased from Amersham (England), expression vector pTrc99A was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden), restriction enzymes were purchased from New England Biolabs (Beverly, MA), SMART™ PCR cDNA Library Construction kit and Marathon™ cDNA Amplification Kit were purchased from Clontech (Palo Alto, CA). *Escherichia coli* NR8052 [Δ(pro-lac), thi-, ara, trpE9777, ung1] (75, 76) and purified recombinant human UNG (UNGΔ84) (64) was provided by Dr. Hans E. Krokan, Institute for Cancer Research and Molecular Biology, Norwegian University of Science and Technology.

Isolation of mRNA

mRNA was isolated from 250 mg cod liver using Oligotex direct mRNA Midi kit (Qiagen), following the instructions in manufacturers protocol.

Preparation of cDNA

cDNA was made from 250 ng of the isolated poly A⁺ RNA using SMART™ PCR cDNA Library Construction kit (Clontech) according to the protocol recommended by the manufacturer. In brief, 1st strand cDNA was made by

combining 250 ng poly A+ RNA with 10 pmol SMART oligonucleotide (5'-TACGGCTGCGAGAAGACGACAGAAGGG-3') and 10 pmol CDS/3' PCR primer (Oligo(dT)30 N -1 N (N = A, C, C, or T; N −1 = A, G, or C)) in a final volume of 5 µl, and incubated at 72°C for 2 min and then placed directly on ice

5   for 2 min to denature the RNA. Then enzyme and buffer were added to the reaction mixture to a final volume of 10 µl, consisting of 50 mM Tris/HCl, pH 8.3, 6 mM MgCl₂, 75 mM KCl, 2 mM DTT, 1 mM dATP, dCTP, dGTP and dTTP respectively and 200 U SuperScript™ II reverse transcriptase (Gibco BRL), and then incubated at 42°C for 1 h. Synthesis of 2nd strand was done by PCR in a

10 final volume of 100 µl, containing 2 µl of the 1st strand reaction as template, 40 mM Tricine/KOH pH 9.2 (25°C), 15 mM KOAc, 3.5 mM Mg(OAc)₂, 3.75 µg/ml BSA, 0.2 mM of dATP, dCTP, dGTP and dTTP respectively, 1U Advantage cDNA Polymerase Mix (Clontech) 0.2 µM 5'-PCR primer (5'-TACGGCTCCGAGAAGACGACAGAA-3') and CDS/3'-PCR primer respectively.

15

PCR amplification was done in a GeneAmp 9700 thermocycler (Perkin Elmer), by 95°C for 1 min followed by 16 cycles of 95°C for 15 sec and 68°C for 5 min.

ds cDNA polishing To 50 µl of the amplified ds cDNA, 40 µg proteinase K was

20 added and incubated at 46°C for 1 h. Proteinase K was inactivated by incubating the mixture at 90°C for 8 min. To blunt end the ds cDNA, 15 U of T4 DNA polymerase was added and incubated at 16°C for 30 min and 72°C for 10 min. Finally the ds cDNA was ethanol precipitated and resuspended in 10 µl H₂O. All tubes were kept on ice if not otherwise stated.

25

Generation of a 300 bp fragment of the cUNG-gene

Degenerated oligonucleotide primers were designed from two conserved regions (GQDPYH and VFLLWG) from known UNG- amino acid sequences. Codon usage for Atlantic cod were also considered when designing the primers.

30 The UNG fragment was generated by PCR with cod liver cDNA as template in a final volume of 50 µl, containing 10 mM Tris/HCl pH 9.0 (25°C), 50 mM KCl, 0.1% Triton X-100, 10 ng cDNA, 0.2 mM dATP, dCTP, dGTP and dTTP

respectively, 2.0 μM upstream primer (5'-GGH-CAR-GAY-CCC-TAY-CA-3') and downstream primer (5'-DCC-CBA-SAG-SAG-RAA-VAC-3')[1] respectively and 2.5 U Taq-polymerase (Promega). PCR was carried out by 94°C for 4 min, 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, and a final
5 extension step of 72°C for 5 min.

The nucleotide symbols used are as follows ; A=Adenine; C=Cytosine; G=Guanine; T=Thymine; D= A+G+T; R=A+G; S=C+G; V=A+C+ G; Y=C+T

10 **DNA sequencing**

DNA sequencing was done with the Amersham Pharmacia Biotech Thermo Sequenace Cy5 Dye Terminator Kit. ALFexpress™ DNA sequencer and ALFwin Sequence Analyzer version 2.10. Gels were made with Reprogel™ Long Read and Reprosel UV-polymerizer. All items were purchased from Amersham
15 Pharmacia Biotech (Uppsala, Sweden).

RACE procedure

Ligation of adaptors to cDNA was done as described in the protocol from the manufacturer. In brief, RACE-adaptors were ligated to cDNA in a total volume of
20 10 μl containing 50 mM Tris-HCl (pH 7.8), 10 mM MgCl2, 10 mM DTT, 1 mM ATP, 0.75 μg cDNA, 2 μM Marathon cDNA adaptor (Clontech), 400 U T4 DNA ligase. The reaction was incubated at 16°C for 16 h, and 5 min at 70°C to inactivate the ligase. Before RACE, the adaptor ligated cDNA was diluted 50 times in TE-buffer and denatured at 100°C for 2 min and placed directly on ice.
25

The sequence deduced from the 300 bp fragment of the UNG-gene was used to design two primers for both 3'- and 5' rapid amplification of cDNA ends (RACE), with a small overlap region between the two fragments generated. Both 3'- and 5'-RACE reactions were done in a volume of 50 μl with 1 μl of
30 diluted cDNA with RACE-adaptors as template, 0.2 μM internal 3'- (5' - TGTACCGACATTGATGGCTTCAAGCAT-3') or 5' - (5'-

CCCATCCGCTTAGATCTCCATGTCCAG-3') RACE primers, r spectively, 0.2 µM AP1-primer (supplied by manufacturer)(5'-CCATCCTAATACGACTCACTATAGGGC-3'), 40 mM Tricine/KOH pH 9.2 (25°C), 15 mM KOAc, 3.5 mM Mg(OAc)$_2$, 3.75 µg/ml BSA, 0.2 mM of each

5   dATP, dCTP, dGTP and dTTP and 1U Advantage cDNA Polymerase Mix (Clontech). Amplification was done in a GeneAmp 9700 thermocycler (Perkin Elmer), 94°C for 30 sec followed by 5 cycles of 94°C for 5 sec and 72°C for 3 min, 5 cycles of 94°C for 5 sec and 70°C for 3 min, and 20 cycles of 94°C for 5 sec and 68°C for 3 min.

10

(Individual bands were purified from an agarose gel, and used as template in a new PCR-reaction with the same conditions as above to generate more DNA.)

Isolation of two different UNG genes

15   Both RACE-fragments generated were sequenced using their respective internal RACE-primers.

Examining the sequence of the 5'-RACE-fragment indicated a double sequence, difficult to read, near the 5'-end of the fragment. However at the end of the fragment only one sequence appeared, due to a long UTR in one of the UNG-

20   sequences but not the other. A new primer complementary to this 5'-end was designed (5'-ATGGAATTCGATTGAGATTGGCGCCTTTGG -3') and a new PCR-reaction was carried out with this, and the 5'-RACE internal primer, with the 5'-RACE fragment as template. The PCR was carried out in a final volume of 50 µl with 40 mM Tricine/KOH pH 9.2 (25°C), 15 mM KOAc, 3.5 mM

25   Mg(OAc)$_2$, 3.75 µg/ml BSA, 0.2 mM of dATP, dCTP, dGTP and dTTP respectively, 1U Advantage cDNA Polymerase Mix (Clontech), 10 ng cDNA as template and 0.2 µM upstream and downstream primers respectively. Amplification was done in a GeneAmp 9700 thermocycler (Perkin Elmer), 94°C for 1 min. followed by 30 cycles of 94°C for 30 sec, 60°C for 1 min and 72°C for

30   1 min.

The fragment was sequenced using the internal RACE-primer, and the sequence was subtracted from the double sequence region described above. A primer complementary to the 5p end of the underlying sequence was designed (consisting of both nucleotides from the SMART-sequence and the remaining

5 UNG-sequence), and a PCR-reaction with this and the internal 5'-RACE-primer was carried out. The new fragment was sequenced as described above. From the two different UNG-sequences in the 5'-RACE-fragment, two final primers (UNG1 and UNG2) were made to isolate the full length UNG1 and UNG2, respectively, using cDNA as template and the same PCR-conditions as

10 described above,

Construction of expression vectors

The catalytic domain of the UNG-gene was cloned in the expression vector pTrc99A, containing a strong trc promoter upstream of a multiple cloning site

15 (77). Several different constructs were made by PCR-amplifying the gene using cDNA as template with upstream and downstream primers containing EcoRI and SalI restriction sites respectively. DNA-fragments were purified, digested with EcoRI and SalI and ligated into the pTrc99A expression vector. In brief, PCR-fragments with restriction sites were generated in a PCR-reaction (50 μl)

20 containing 40 mM Tricine/KOH pH 9.2 (25°C), 15 mM KOAc, 3.5 mM Mg(OAc)$_2$, 3.75 μg/ml BSA, 0.2 mM of dATP, dCTP, dGTP and dTTP respectively, 1U Advantage cDNA Polymerase Mix (Clontech), 10 ng cDNA as template and 0.2 μM upstream and downstream primers respectively. Amplification was done in a GeneAmp 9700 thermocycler (Perkin Elmer), 94°C for 1 min followed by 30

25 cycles of 94°C for 30 sec, 60°C for 1 min and 72°C for 2 min, and a final extension step of 72°C for 5 min. Individual bands were purified from agarose gel, and used as template in a new PCR-reaction, with the same conditions as described above, to generate more DNA. DNA was purified using Quiaquick PCR purification kit (Millipore) as described by manufacturer, and eluted in TE-

30 buffer, pH 8.0. Restriction enzyme digestion was done in a final volume of 30 μl containing 1 μg insert DNA or 0.25 μg pTrc99A vector, 6 mM Tris/HCl, pH 7.9, 6 mM MgCl$_2$, 150 mM NaCl, 1 mM DTT (BufferD, Promega) and 3U of EcoRI and

SalI. The mixtures were incubated at 37°C for 3 h, followed by two times phenol/chloroform extraction, ethanol precipition and resuspended in 5 µl H$_2$O. Ligations were performed in a total volume of 10 µl containing 50 mM Tris-HCl (pH 7.8), 10 mM MgCl$_2$, 10 mM DTT, 1 mM ATP, 100 U T4 DNA ligase, 250 ng

5   vector DNA and 1 µg insert DNA, and incubated at 16°C for 16 h.

Competent *E. coli* JM105 (200 µl) was transformed with ligation mixes and grown on LB+ plates containing 100 µg/ml ampicillin at 37°C. Plasmid DNA was reisolated from positive clones and transformed in *F. coli* NR8052 used for

10   expression of recombinant UNG.

Four different constructs were made rcUNGΔ74 and rcUNGΔ74o and rcUNGΔ81 and rcUNGΔ81o where 74 and 81 of the N-terminal amino acids were removed, respectively. These have the same length as the human Δ77

15   and Δ84, respectively (64). The Δ74o and the Δ81o constructs have the codons encoding arginine 87 and 88 optimized for expression in *E.coli*, by changing them from AGA to CGT. All constructs were made by PCR as described above, using the following primers and template :

20   rcUNGΔ74 : UDGL77 (5'-AACCATGGAATTCGCAAAAGCAACGCCTGCA- 3 ')
and UDGEND2 (5'-
GAGCICGTCGACTTAGAGTGCCTCTCCAGTTTATAGG- 3')and 10ng cDNA
as template.

25   rcUNGΔ81;   UDGL84 (5'- ACCATGGAAI ICTTCGGAGAGACTTGGAGAAGA
– 3') and UDGEND2 and 10 ng cDNA as template.

rcUNGΔ74o : (5'-
30   ATGGAATTCGCAAAAGCAACGCCTGCAGGTTTCGGAGAGACTTGGCGTCG
TCAG – 3') and UDGEND2 and 1 ng rcUNGΔ81o as template.

rcUNGΔ81o : (5'-
ATGGAATTCTTCGGAGAGACTTGGCGTCGTGAGCTGGCTGC – 3') and
UDGEND2 and 10 ng cDNA as template.

5 **Small scale expression of uracil-DNA glycosylase**

Optimization of expression were done in 1l baffled erlenmeyer flasks with 100
ml LB+ medium with 100 µg/ml ampicillin, inoculated with 5 ml of preculture.
Cells were induced with 1 mM of IPTG at OD600 = 2.0, and induced at various
length as indicated in figure legends. Expression was examined using various
10 temperatures (20°C, 25°C, 30°C and 37°C).

The conditions above were also used to induce expression at various IPTG-
concentrations (1 mM, 0.5 mM, 0.1 mM and 0.01mM).

15 **Fermentation conditions**

Fermentation was done in a 10 l Chemap CF 3000 fermentor (Switzerland). A
200 ml preculture of *E.coli* NR8052 with the pTrc99A oΔ84 construct was
inoculated to 7 l of LB-medium supplemented with 20 mM glucose and 100
µg/ml ampicillin. Cells were grown to an OD600 of 2.0 and induced for 8 h with
20 1 mM IPTG. Additional glucose (3 x 50 ml of 20% glucose (W/v)) was
supplemented during the fermentation to avoid glucose starvation. Cells were
harvested and centrifuged at 10.000 g for 10 minutes. The cell paste was frozen
at -70°C.

25 **Purification of recombinant cod UNG**
Crude extract

From the fermentation, 4 l of the *E. coli* NR8052 cells (68 g wet weight) were
resuspended in 400 ml of extraction buffer (25 mM Tris/HCl, 10 mM NaCl, 1 mM
EDTA, 1 % glycerol, 1 mM DTT, 1 mM PMSF pH 8,0). The cells were disrupted
30 by subjecting them five times through the Nebulizer using 100 psi of nitrogen.
The extract was centrifuged 25.000 g for 10 min, and the supernatant removed.
The pellet was resuspended in 100 ml of buffer A, and re-centrifuged as

described above. The supernatants were combined and filtrated through glasswool (460 ml).

Protamine sulphate

5 To the crude extract (460 ml), 60 ml of 2 % protamine sulphate in buffer A (25 mM Tris/HCl, 10 mM NaCl, 1 mM EDTA, 1 % glycerol, pH 8.0) was added and incubated at 4°C for 5 min with stirring. The solution was centrifuged at 25.000 g for 10 min, and the supernatant was removed, 510 ml (fraction 1).

10 ## Q/S-sepharose

The protamine sulphate fraction was applied on a Q-sepharose column (5.0/10) coupled with a S-sepharose column (2.6/10), both equilibrated in buffer A, using a flow rate of 10 ml/min Then the columns were washed with 750 ml buffer A, and the Q-sepharose column was then removed. The S-sepharose column was

15 washed with an additional 550 ml buffer A + 60 mM NaCl, and a gradient of 60 to 400 mM NaCl in buffer A was applied to elute the column, using a flow rate of 5 ml/min. Fractions of 10 ml were collected, and fractions containing UNG-activity were pooled (fraction 2, 115 ml).

20 ## Blue sepharose FF

Fraction 2 was diluted two times in buffer A, and directly applied to a blue sepharose column (1.6/5.0), with a flow rate of 4 ml/min. The column was then washed with 30 ml of buffer A and 60 ml of buffer A + 110 mM NaCl, and eluted with 60 ml buffer A + 0.7 M NaCl. UNG containing fractions were pooled

25 (fraction 3, 24 ml).

Superdex 75

Fraction 3 was concentrated to 3 ml using a Ultrafree 15 unit, (Millipore) and applied to the superdex 75 column (2.6/60) equilibrated in buffer A + 0.15 M

30 NaCl. A flow rate of 2 ml/min was used, and fractions of 5 ml were collected. Fractions containing UNG-activity were pooled (fraction 4, 30 ml).

Source 15Q

The Superdex 75 fraction was diafiltrated in an Ultrafree 15 unit (Millipore) and approximately 2/3 of the Superdex 75 fraction was applied to the Source 15Q column (2.6/3.0) equilibrated in buffer A at room temperature. The column was

5 washed with 60 ml buffer A + 60 mM NaCl, and then a gradient from 60 to 210 mM NaCl in buffer A was applied to elute the column. Fractions were collected on ice, and UNG-containing fractions were pooled (UNG-activity eluted between 105 to 145 mM NaCl).

10 **RESULTS**

Nucelotide sequences as well as one-letter code amino acid sequences for cUNG1 and cUNG2, respectively are given in the enclosed Sequence listings, SEQ:ID:NOS 1 and 2.

15 **Expression**

Expression of rcUNG by the pTrc99A-Δ81o construct in 7 liter scale fermentation yielded a total of 413,480 Units of rcUNG contained in the crude extract.

20 **Purification of recombinant cod UNG**

Purification of recombinant cUNG (Δ81o) (rcUNG) is summarized in table 1. From a 4 l of the fermentation batch, 4.8 mg of recombinant UNG was purified to apparent homogeneity, and the molecular weight was determined to 28 kD by SDS-PAGE. The specific activity of the enzyme was determined to 30,092

25 U/mg using the nick-generated substrate with standard assay conditions. This preparation was found not to contain detectable levels of DNA-endodeoxy ribonucleases or - exodeoxy ribonucleases according to standard methods.

30

Example 3

Measurement of residual activity/r activati n after thermal inactivation

11.5 Units of rcUNG (approximately 0.38 µg) in 50 µl "Taq-buffer" : (10mM tris-HCl (pH=9.0 at 25 °C), 50mM KCl, 0.1% Triton X-100) was inactivated for 10.

5 min at 94 °C, and divided into two equal parts. One part was kept at 4 °C and the other part was kept at 25 °C before residual activity was measured at 1 and 16 hours after inactivation, using 15 µl undiluted inactivated mixture in a standard assay. Before inactivation, 2µl was taken out and diluted 1:2000 before measuring the activity (100% control). I he results showed that the

10 inactivated enzyme did not cause release of radio labelled uracil from the substrate that was above the detection limit (blank control + 2x standard deviation). At these conditions, the 100% control would yield 7.000.000 cpm, while 2x background standard deviation was 46 cpm. Consequently, the residual activity of rcUNG was less than 0.0006% of initial activity after thermal

15 inactivation at these conditions There was neither any significant reactivation of the enzyme after 16 hours at either 4 °C or 25 ºC.

The results show that rcUNC has no practical residual activity after thermal inactivation and that it does not reactivate. The enzyme rcUNG is therefore

20 different from the previously isolated UNG from the marine bacterium, BMTU (45) which was shown to have a small degree of reactivation or residual activity after thermal inactivation.

Example 4

25 **Carry - over prevention**

It is tested whether cUNG is effective to degrade contaminating uracil-containing DNA in a carry-over prevention reaction.

Method

30 As a contaminant, 0.5 ng of uracil containing template DNA (761 bp fragment generated from cationic trypsinogen from Atlantic salmon (*Salmo salar*) (61) was added to the PCR-mix in a final volume of 50 µl, containing 10 mM Tris/l ICl pH 9.0 (25°C), 50 mM KCl, 0.1% Triton X-100, 10 ng cDNA, 0.2 mM dATP, dCTP, dGTP and dTTP respectively, 2.0 µM upstream- (OP5) and downstream

35 primer (NP2) respectively and 1.0 U Taq-polymerasc (Promega). UNG (4 x 10^{-3}

or 1./ x 10^{-2} U) was added to the PCR-mix and incubated at ambient temperature (RT) for 10 minutes.

As negative controls, one PCR reaction mixture contained template with
5   thymidine replacing uracil, and in one mixture water replaced UNG. Also, as a positive control 1.7 x 10^{-3} U UNG from *E. coli* was used, instead of rcUNG.

Then PCR was carried out by 94°C for 4 min, 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. After the PCR the products were analyzed by
10   agarose gel electrophoresis.

PCR primer sequences:

OP5:
5'-TCTCTCGAGAAAAGAGAGGCTGAAGCTCCCATTGACGATCAGGATGA-3'

15

NP2:
5'-GTAGAATTCGGATCCATGTCTCCTCCAGTCTAGAT-3'

Result
20   The result of the experiment according to the example is shown in fig. 9. The uracil-containing templates were degraded by UNG in all PCR reactions while the reactions either without UNG or with thymine-containing templates yielded the expected PCR product. The results in fig. 9 are shown as a figure of an agarose gel. The results show that rcUNG is effective to degrade contaminating
25   uracil-containing DNA in a PCR reaction.

Table 1: Purificati n f Atlantic cod liver uracil-DNA glycosylase (rcUNG).

Step	Volume (ml)	Activity . (U/ml)	Total activity (U)	Protein conc (mg/ml) b	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification Fold
Crude extract	2000	0.086	172	4	8000	0,021	100	1
Q-Sepharose FF	2340	0,073	1/0	1,66	3864	0.044	99	2
S-Sepharose FF	55	1,796	98,8	0,155	8,53	11,6	58	540
Heparin Sepharose	20	2,775	66,6	0,081	1,62	34,3	32	1597
Poly-U Sepharose	70	0,446	31,2	ND c	ND	ND	16	ND
Superdex75	3	1,138	3,41	0.003	0,009	379	2	17679

a) Enzyme activity was measured as described under standard assay in material and methods using nick-substrate, in Example 2.

b) Protein concentration was determined as described in material and methods.

5   c) Protein concentration was to low to determine.

Table 2: Substrate specificity ssDNA versus dsDNA.

Substrate	cpm	% activity	Fold
dsNick	586	54	1,0
ssNick	1078	100	1,8
dsPCR	1158	52	1,0
ssPCR	2214	100	1,9

The present results show for the first time isolation, and use In carry-over
10   prevention in DNA copying reactions, of an UNG from a cold-adapted
eukaryotic organism. The previously known UNG enzymes isolated from E.coli
(60) or BMTU (45), for use in PCR reactions are of prokaryotic origin.

The cUNG is completely inactivated when heated above 60 °C and the
15   deactiviation is completely irreversible (as there is no detectable residual
enzyme after heat treatment). This quality makes cUNG a better candidate as
an enzyme for use in carry-over prevention in DNA-copying reactions (PCR;

LCR etc.) compared to enzymes previously known in this field, as *E.coli* UNG or UNG from BMTU, which were not completely and irreversibly inactivated after heat treatment. Because of the complete and irreversible deactivation of cUNG by the heat treatment normally performed in PCR reaction cycles there is

5 no detectable residual enzyme after heat treatment. Then there will be no unwanted degradation of the DNA product in the upscaling reaction by residual UDG enzyme activity and of course there will be no need to add enzyme inhibitor to avoid degradation of DNA by such residual UDG enzyme activity.

10 It is shown that the recombinant cUNG (rcUNG) has the same qualities/functions as the cUNG isolated from cod liver. Cod liver contains only small amounts of cUNG. When cUNG is produced recombinantly, larger quantities of cUNG can be made compared to the amount of cUNG produced by extraction from cod liver.

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